REMARKS

Claims 5, 8, 10, 13-17, 19, 24, and 37-39 have been cancelled. Claims 1-4, 6-7, 9, 11-12, 18, 20-23, 25, and 27-32 have been amended. New claims 40-66 are added. Claims 1-4, 6-7, 9, 11-12, 18, 20-23, 25-36, and 40-66 are now pending in this application. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

The amended claims include the feature that 'low level or unstable RNA is quantified' from 'whole blood'. It has been further indicated in the claims that 'real in vivo levels' are determined or analyzed. Support for the claim amendments is found, for example, at page 3, lines 6-7 of the present specification. Other minor changes have also been introduced.

Claim objections

Claims 32-36 are objected to as being improperly multiply dependent. With this amendment, multiple dependencies have been removed. Accordingly, the objection may be withdrawn.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 5, 8, 10, and 13 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

This ground of rejection is now moot in view of Applicants' cancellation of these claims.

Rejection under 35 U.S.C. § 102(b) (Hamel)

Claims 1-4, 6, 7, 12 and 20-22 are rejected under 35 U.S.C. § 102 (b) as being anticipated by Hamel, et al. (Journal of Clinical Microbiology, Feb. 1995, Vol. 33, No. 2, page 287-291).

Applicants respectfully traverse the rejection as follows. The method of the present invention is clearly distinct from the method disclosed by Hamel et al. (1995) in at least three different aspects. These are discussed in the paragraphs below.

1/ Hamel et al. teach a method wherein RNA is analyzed using gel analysis or southern hybridization (see for instance Fig 1); no quantification has been performed.

2/ In addition, Hamel et al. analyzes RNA from the bovine viral diarrhea virus. It is of general knowledge that RNA from viruses are present at high levels and are highly stable. Consequently, in Hamel et al. no low level or unstable RNA is analyzed.

3/ Furthermore, Applicants respectfully submit that vortex mixers, microcentrifuges, micropipettes and the thermocycler should be seen as different and clearly distinct from the automated setup disclosed in the present application. Indeed, although *devices* are used to perform the manipulations, Applicants submit that the methods performed by Hamel et al. should be considered as hands-on methods. Contrarily, the methods referred to in the present application relate more to hands-off systems in which full automated systems are used. The application indicates that the automated system should be seen as a walk-away system (p.10, line 3 of the application as originally filed). Examples of such automated devices are given in the application as originally filed. The devices may be full automatic devices such as MagNA Pure LC Instrument (Roche Diagnostics), the AutoGenprep 960 (Autogen), the ABI PrismTM 6700 Automated Nucleic Acid Workstation (Applied Biosystems), WAVE® Nucleic Acid Analysis System with the optional WAVE® Fragment Collector FCW 200 (Transgenomic) and the BioRobot 8000 (Qiagen) (see for instance p.5, lines 1-6 of the application as originally filed). The automated devices referred at in the present claims are thus clearly different from the devices used by Hamel et al.

Accordingly, as Hamel, et al. do not teach all of the limitations of the presently claimed invention, Applicants respectfully submit that Hamel, et al. do not anticipate the present claims.

Furthermore, Applicants respectfully submit that the claimed invention is non-obvious over Hamel, et al. Hamel et al. do not suggest or lead the skilled person to the concept of the present invention. The document does not indicate that "real in vivo levels of low-level or unstable RNA from whole blood" may be quantified in an extremely accurate way using a more automated approach. A skilled person may not derive from Hamel et al. that a more automated method of the disclosed method may be used to accurately quantify said low-level RNA. It is only through the disclosure of the technical characteristics in the present application that a skilled person may arrive at the invention as presently claimed..

The claimed method allows the quantification of low copy or unstable RNA present in a cell at the moment of sampling in an extremely accurate way. For this reason, the claims indicate

that "*real in vivo levels*" are analyzed. The new methodology provides *a sensitive and accurate way to quantify low level or unstable nucleic acids* in biological samples which was not possible using prior art methods (p.37, lines 16-18 of the application as originally filed). Only by applying the method of the present invention correct (or real) *in vivo* levels of low level or unstable nucleic acid transcripts in blood may be determined.

Prior art methods aiming at the analysis of RNA from whole blood samples consist of the use of an RNA-stabilization reagent in combination with a spin column system (see PAX isolation kit analyzed in the present application as reference prior art method) or in combination with the classical RNA isolation procedures (see Hamel et al.).

The problem to be solved by the present invention may be regarded as providing a more reliable and reproducible (= *accurate*) method for the quantification of *real* in vivo *levels* of low level or unstable RNA transcripts from a biological sample. The application focuses on whole blood as the biological sample.

The solution given by the present invention is the provision of an automated method which unexpectedly allows to quantify accurately and with an extreme precision low level or unstable RNA in a blood sample. This automation should not be seen as a 'general automation' of a method wherein the fast analysis of a large number of samples is aimed at. Contrarily, as explained above the aim of the present invention is to improve accuracy of the quantification of low level or unstable RNA.

M.P.E.P. 2145 states that the "totality of the prior art must be considered, and proceeding contrary to accepted wisdom in the art is evidence of nonobviousness. *In re Hedges,* 783 F.2d 1038, 228 USPQ 685 (Fed. Cir. 1986)...Furthermore, "[k]nown disadvantages in old devices which would naturally discourage search for new inventions may be taken into account in determining obviousness." *United States v. Adams,* 383 U.S. 39, 52, 148 USPQ 479, 484 (1966).

In the paragraphs hereunder we further illustrate that for several reasons *one of ordinary skill in the art would not tend to automate existing methods comprising the use of RNA stabilization reagents*. Proof of the invention is also discussed.

As indicated above, prior art methods aiming at the analysis of RNA from whole blood samples consist of the use of an RNA-stabilization reagent in combination with a spin column system, or, in combination with the classical RNA isolation and analysis steps. None of these are

'walk away systems'. As indicated below, a skilled person would never automate one of said existing methods in order to accurately quantify low copy or unstable RNA. Reasoning otherwise is based on impermissible hindsight.

1. A skilled person in the art would not automate existing methods consisting of the use of RNA-stabilization reagent in combination with a spin column system.

For the PAXgene™ Blood RNA System there is an obligatory combination between the PAXgene™ Blood RNA Tube and the PAXgene™ Blood RNA Kit. There is no reason why the person skilled in the art would only use parts of this PAXgene™ Blood RNA System and develop a new method therefrom (see p.4, lines 30-34 of the application as originally filed). In addition, according to the PAXgene manufacturer, RNA isolation from PAXgene tubes requires their RNA extraction kit, and therefore is not supposed to work using other conditions (p.8, lines 3-5 of the Handbook; copy attached as Attachment A).

2. A skilled person in the art would not automate existing methods consisting of the use of RNA-stabilization reagent in combination with the classical RNA isolation steps to develop an accurate method to quantify low level or unstable RNA from whole blood.

As indicated above, 'automation' of a method is normally applied to increase the speed of hands-on steps and of the number of samples manipulated.

Contrarily, as explained above the aim of the present invention is to improve accuracy of the RNA quantification. Accuracy may only be guaranteed when the method is applied by a scientist who works accurately and looks at the samples with a critical eye. It is known for a skilled person that looking at the samples when treating samples is of primary importance. Indeed during manipulation steps samples sometimes behave very strangely, even if manipulation conditions have been set for routine manipulations. Therefore, a skilled person would NEVER, when aiming at the accurate analysis of low level or unstable RNA in a blood sample try to automate the hands-on steps performed.

Furthermore, a skilled person knows that automation, inherently involves the use of smaller volumes. A skilled person would never use small volumes when there is a need to quantify low level or unstable RNA. Consequently, in order to quantify accurately low level or unstable RNA in a sample the skilled person would never use automated systems.

Applicants further wish to indicate that the blood samples used are very *precious samples*. Indeed, once a sample is taken, there is only a limited possibility to try to test new procedures. Therefore, a skilled person would, especially when working with human blood samples, try to be cautious and follow carefully the conditions which were previously found to work. He would never play with conditions to find a more optimal method, in particular when not expecting a high chance of success.

We further note that at the time of the filing of the present application, Catrimox14 (used by Hamel et al.) had completely disappeared from the market for several years, making it nearly impossible to further work on the method disclosed. No alternatives, except for the PAX system, were available. Furthermore, said PAX system may be considered as a black-box-system, as no details of the compositions used in said system are given by the supplier.

A person skilled in the art would thus not be able to deduce the newly claimed subject matter based on the cited prior art documents separately, by combining said documents or in combination with the common general knowledge, without using hindsight. It is only through the specific and selective testing, as performed by the present inventors, that it became clear that real in vivo quantities of low-level or unstable RNA can only be obtained through the automation of the existing methods which allow the stabilization of nucleic acids in samples using an automated setup for RNA isolation and analysis.

The claims as amended are thus a result of inventive thoughts and curiosity of a creative scientist, and not from routine optimization by one of ordinary skill in the art . The person skilled in the art should be presumed to be an ordinary practitioner aware of what common general knowledge in the art at the relevant date. He will thus not try to explore experimental conditions from which no results may be expected.

Applicants also note that at the time the inventors published the method disclosed in the present application, many scientists were surprised by these findings. Dr. Stordeur, who is one of

the inventors, has frequently been invited to write book chapters and peer reviews, and to present data at important meetings to explain his particular findings relating to the presently claimed invention.

For the reasons given above, Applicants respectfully submit that the presently claimed invention is patentable over Hamel, et al.

As discussed above, before the filing date of the present application, there existed no methodology to provide a sensitive and accurate way to quantify low level or unstable nucleic acids in biological samples.

The inventors found that this problem can be avoided when combining the use of a surfactant to stabilize RNA with automated methods to isolate the nucleic acids and further analysis. The inventors found that by applying such an automated system low level or unstable RNA can be quantified with an extreme high accuracy which could not be obtained with prior art methods.

For instance Figure 6 of the application clearly indicates that the copy number of low level RNA (in this case IL-1β) may be determined in an accurate way (r=0.987). Please note that raw copy numbers are determined without any correction of the signals obtained. Alternatively, such a correction or standardization may also be performed by for instance measuring the OD of the sample. In the method of the present invention, there is thus no absolute need to measure mRNA concentration for expression of the results using the method of the invention (p.38, lines 22-24 of the application as originally filed). That such an accuracy may be obtained for small samples of 20-200 µl of blood implies that such accuracy should be found when applying a larger volume of blood.

In addition, the reproducibility studies presented in Table 3 illustrate that the method according to the present invention is a more reliable and accurate method compared to the system applied using the Qiagen mRNA extraction method as suggested by the PAXgene^{TM} Blood RNA kit. The inventors found thus surprisingly that the combination proposed in the method of the present invention is possible and that this combination provides a powerful technique for the accurate mRNA quantification from biological samples. Applicants note again that at the time of the filing of the present application, the PAX system was the only commercially available system to isolate RNA from blood. As indicated above, starting from said system, a skilled person would

never tend to change the conditions used therein and to derive the method of the present invention.

Furthermore, the inventors and also other scientists experienced that the accuracy of the method of the present invention is not comparable to the accuracy found when applying the method as disclosed in the prior art. The latter method works without sensitivity and accuracy. There is no possibility to detect any differences or changes induced by in vivo situations.

An accurate RNA quantification is thus only possible when applying the method of the present invention. Using the prior art methods it is not possible to see small RNA variations in blood, even when using large sample volumes.

In view of Applicants' amendments and arguments presented above, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 103(a) (Winer, Hamel)

Claims 9 and 11 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Winer, et al. (Analytical Biochemistry 270, 41-49 (1999) in view of Hamel, et al. (Journal of Clinical Microbiology, Feb. 1995, vol. 33, No. 2, p. 287-291).

The Examiner concedes that Winer does not teach the method according to claim 1 or 2 (Office Action, page 10, line 3) and relies upon Hamel, et al. as teaching the methods of claims 1 or 2. However, for the reasons given above (which are incorporated by reference here), Applicants argue that Hamel, et al. do not anticipate or render obvious the claimed invention. As Winer, et al. do not teach the invention of claims 1 or 2, as conceded in the Office Action, Winer, et al. cannot correct the deficiencies of Hamel, et al., discussed above. Accordingly as claims 9 and 11 depend from claim 1, which is neither taught nor suggested by the cited references taken separately or together, Applicants respectfully submit that claims 9 and 11 are patentable over Winer, et al. in view of Hamel, et al.

In view of Applicants amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 103(a) (Hamel, Walker)

The Examiner has rejected claim 18 as being unpatentable over Hamel, et al. (Journal of Clinical Microbiology, Feb. 1995, vol. 33, No. 2, p. 287-291) in view of Walker (J. Biochem Molecular Toxicology, vol. 15, No. 3, 2001). The Examiner asserts that it would have been

obvious to one of ordinary skill in the art to use the method of Hamel to quantify DNA as well as RNA in view of Walker. However, since claim 18 depends from claim 1, which is neither taught nor suggested by Hamel, et al as discussed above, the invention defined in claim 18 is also patentably distinguished from the references, alone or in combination. Applicants respectfully request the withdrawal of the rejection.

Rejection under 35 U.S.C. § 103(a) (Kammula, Hamel)

The Examiner has rejected claim 23 and 25-31 as being unpatentable over Kammula, et al. (J. Natl Cancer Inst 2000, 92: 1336-44) in view of Hamel, et al. (Journal of Clinical Microbiology, Feb. 1995, vol. 33, No. 2, p. 287-291). The Office Action concedes that Kammula "does not teach certain steps in the method of claim 1" (Office Action, page 17, line 1) and relies upon Hamel et al. for this teaching. As discussed above (and incorporated here by reference), Hamel does not teach or suggest the invention of present claim 1. This deficiency is not corrected by Kammula.

The Examiner asserts that it would have been obvious to one of ordinary skill in the art to use the method of Hamel to isolate the mRNA in the method of Kammula. However, since claims 23 and 25-31 depend ultimately from claim 1, which is neither taught nor suggested by Hamel, et al. and Kammula, et al, taken separately or together, the invention defined in claims 23 and 25-31 is also patentably distinguished from the references, alone or in combination. Applicants respectfully request the withdrawal of the rejection.

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Aug. 13, 2007

By: Che S. Chereskin

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ATTACHMENT A

PAXgene™

Blood RNA Kit Handbook

For isolation of cellular RNA from whole blood

Important: To be used only in conjunction with PAXgene Blood RNA tubes

For research use only

Not for use in diagnostic procedures

February 2004

PreAnalytiX
A QIAGEN / BD Company

PreAnalytiX
PreAnalytiX GmbH
Feldbachstrasse
CH – 8634 Hombrechtikon
Switzerland

PreAnalytiX Distributors

PreAnalytiX products are manufactured for PreAnalytiX by QIAGEN or BD and are distributed for PreAnalytiX by QIAGEN. Products cannot be ordered at PreAnalytiX GmbH.

Please see the last page for contact information for your local PreAnalytiX distributor.

Contents

Kit Contents

PAXgene™ Blood RNA Kit (50)	762134
Number of preps	50
Buffer BR1 (resuspension buffer)	20 ml
Buffer BR2 (binding buffer)*	18 ml
Buffer BR3 (wash buffer)*	45 ml
Buffer BR4 (wash buffer)†	11 ml
Buffer BR5 (elution buffer)	5 ml
RNase-free water	280 ml
Proteinase K	2 x 1.4 ml
PAXgene Blood RNA Spin Columns	50
Processing tubes (2 ml)	200
Secondary Hemogard® closures	50
Elution tubes (1.5 ml)	50
Handbook	1

* *Not compatible with disinfecting reagents containing bleach. Contains guanidine thiocyanate which is harmful. Take appropriate safety measures and wear gloves when handling.*

† *Buffer BR4 is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.*

Storage

The buffers and PAXgene Blood RNA Spin Columns can be stored dry at room temperature (15–25°C) for up to 1 year.

The PAXgene Blood RNA Kit contains a novel, ready-to-use Proteinase K solution that is stable for at least one year after delivery when stored at room temperature. To store for more than one year, we suggest storing the Proteinase K at 2–8°C.

Product Use Limitations

For research use only. Not for use in diagnostic procedures. The performance characteristics of this product have not been fully established.

Product Warranty and Satisfaction Guarantee

PreAnalytiX guarantees the performance of all products in the manner described in our literature. The purchaser must determine the suitability of the product for its particular use.

PreAnalytiX products are manufactured for PreAnalytiX by QIAGEN or BD and are distributed for PreAnalytiX by QIAGEN.

Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN, the distributor of PreAnalytiX products, will replace it free of charge or refund the purchase price. PreAnalytiX reserves the right to change, alter, or modify any product to enhance its performance and design. If a PreAnalytiX product does not meet your expectations, simply call your local QIAGEN Technical Service Department or other PreAnalytiX distributor listed on the last page of this handbook. As the distributor of PreAnalytiX products, QIAGEN will credit your account or exchange the product — as you wish.

Technical Assistance

Technical assistance with PreAnalytiX products is provided by QIAGEN, the distributor for PreAnalytiX. The Technical Service Departments at QIAGEN are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology. If you have any questions or experience any difficulties regarding the PAXgene Blood RNA System, please contact one of the QIAGEN Technical Services Departments listed on the last page of this handbook.

PreAnalytiX customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at PreAnalytiX. We therefore encourage you to contact us through QIAGEN's Technical Service Departments if you have any suggestions about product performance or new applications and techniques.

Safety Information

To avoid the risk of infection (e.g., from HIV or Hepatitis B viruses) or injury when working with biological and chemical materials, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information about chemicals, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at **www.preanalytix.com/rna_msds.asp** where you can find, view, and print the MSDSs for this kit.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffers BR2 and BR3 contain guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Sample-preparation waste, such as supernatants from centrifugation steps in the RNA purification procedure, are to be considered potentially infectious. Before disposal, the waste must be autoclaved or burned to destroy any infectious material.

The following risk and safety phrases apply to the components of the PAXgene Blood RNA Kit:

Buffer BR2

 Xn

Contains guanidine thiocyanate: harmful. Risk and safety phrases:* R20/21/22-32, S13-26-36-46

Buffer BR3

Contains ethanol: flammable. Risk phrase:* R10

Proteinase K

 Xn

Contains proteinase K: harmful, irritant. Risk and safety phrases:* R36/37/38-42/43, S23-24-26-36/37

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany, Tel.: +49-6131-19240

** R10: Flammable; R20/21/22: Harmful by inhalation, in contact with skin and if swallowed; R32: Contact with acids liberates very toxic gas; R36/37/38: Irritating to eyes, respiratory system and skin; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink, and animal feedingstuffs; S23: Do not breathe vapor; S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protective clothing and gloves; S46: If swallowed, seek medical advice immediately and show this container or label.*

Introduction

Collecting whole blood is the first step in many molecular assays for studying cellular RNA in human white blood cells. A major problem in such experiments is the instability of the cellular RNA profile in vitro. Studies at PreAnalytiX have shown that the copy numbers of individual mRNA species in whole blood can change more than 1000-fold during storage or transport at room temperature. This is caused both by rapid RNA degradation and by induced expression of certain genes within minutes after the blood is drawn. Such changes in the RNA expression profile make reliable studies of gene expression problematic. A method that preserves the RNA expression profile during and immediately after blood is drawn is therefore essential for accurate analysis of gene expression in human whole blood.

Comparison of PAXgene Blood RNA System with a Traditional Blood Collection Method

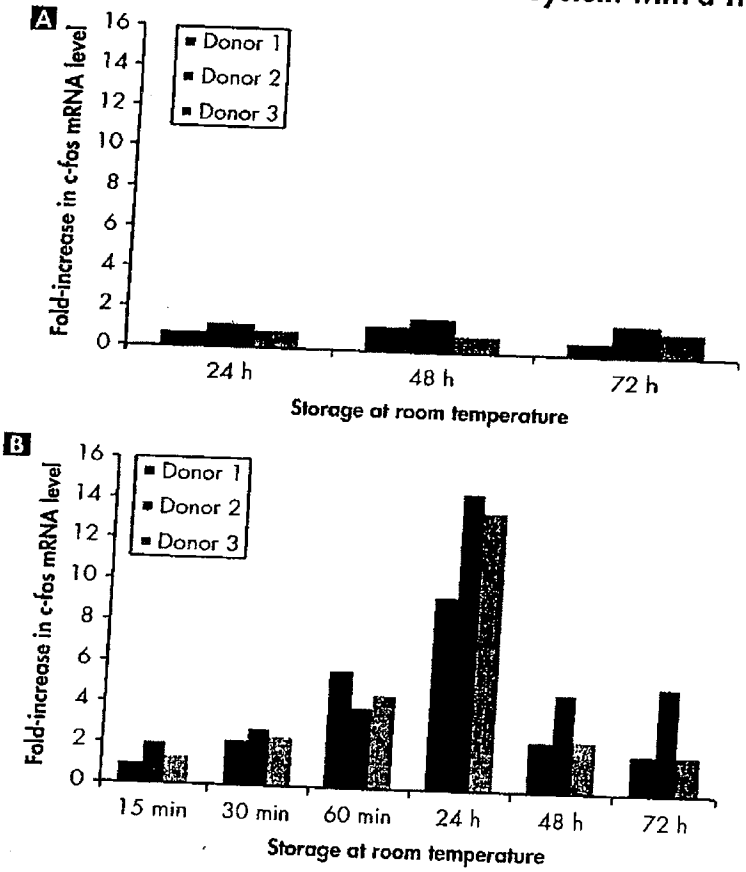


Figure 1. **A** *Blood was collected from 3 donors into PAXgene Blood RNA Tubes and stored for 2, 24, 48, and 72 hours at room temperature before RNA was purified using the PAXgene Blood RNA Kit with the DNase digestion step. Levels of c-fos mRNA were quantified by real-time RT-PCR and normalized to levels of 18S rRNA. The graph shows fold-changes in c-fos mRNA levels with regard to the levels at 2 hours (t = 0).* **B** *Blood was collected from the same 3 donors into EDTA-containing tubes and stored at room temperature. Aliquots were removed at time-points from <5 minutes up to 72 hours for RNA purification using a traditional phenol–chloroform procedure. Purified RNA was cleaned up using the QIAGEN RNeasy® procedure to remove chemical contaminants, and then treated with DNase. Levels of c-fos mRNA were quantified by real-time RT-PCR and normalized to levels of 18S rRNA. The graph shows fold-increases in c-fos mRNA levels with regard to the levels at <5 minutes (t = 0).*

PreAnalytiX has developed the PAXgene Blood RNA System, which enables the collection, stabilization, and transportation of whole blood specimens, and the rapid and efficient isolation of cellular RNA. The system requires the combined use of PAXgene Blood RNA Tubes (patented technology) for blood collection and RNA stabilization, and the PAXgene Blood RNA Kit for RNA isolation. PAXgene Blood RNA Tubes contain a proprietary blend of reagents based on a patented RNA stabilization technology. Components of this blend protect RNA molecules from degradation by RNases and prevent induction of gene expression (Figure 1). The blend also promotes concentration of RNA during the first centrifugation step in the PAXgene Blood RNA purification procedure without the need to open the tube. Silica-gel–membrane technology is then used to isolate high-quality cellular RNA that is ideal for use in sensitive downstream applications.

PAXgene Blood RNA Tubes are intended for the collection of whole blood and the stabilization of the cellular RNA profile. The cellular RNA profile may remain stable for up to 5 days at room temperature (18–22°C). The actual duration of RNA stabilization may vary depending upon the species of cellular RNA and the downstream application used, and should be evaluated accordingly. The performance characteristics of PAXgene Blood RNA Tubes have not been established.

Principle and procedure

The PAXgene Blood RNA Kit allows the isolation of total RNA from 2.5 ml human whole blood samples collected in PAXgene Blood RNA Tubes. The procedure is simple (see flowchart, page 11). Isolation begins with a centrifugation step to pellet nucleic acids in the PAXgene Blood RNA Tube. The pellet is washed, resuspended, and incubated in optimized buffers containing Proteinase K to digest proteins. A second centrifugation step is carried out to remove residual cell debris, and the supernatant is transferred to a fresh microcentrifuge tube. Ethanol is added to adjust binding conditions, and the lysate is applied to a PAXgene RNA spin column. During a brief centrifugation, RNA is selectively bound to the silica-gel membrane of the spin column as contaminants pass through. Remaining contaminants are removed in three wash steps, and pure RNA is then eluted in Buffer BR5.

Generally, DNase digestion is not required for RNA purified with the PAXgene Blood RNA Kit, since PAXgene silica-gel–membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal is recommended for RNA applications that are sensitive to DNA contamination. In these cases, residual DNA can be removed either by using the RNase-Free DNase Set (QIAGEN, cat. no. 79254) for on-column DNase digestion, or by treating the eluted RNA with DNase.

Using the PAXgene Blood RNA System, typical yields of RNA isolated from 2.5 ml human whole blood are between 4 and 10 µg. However, the yield is highly donor-dependent, and in some cases, higher or lower yields may be achieved (Figure 2).

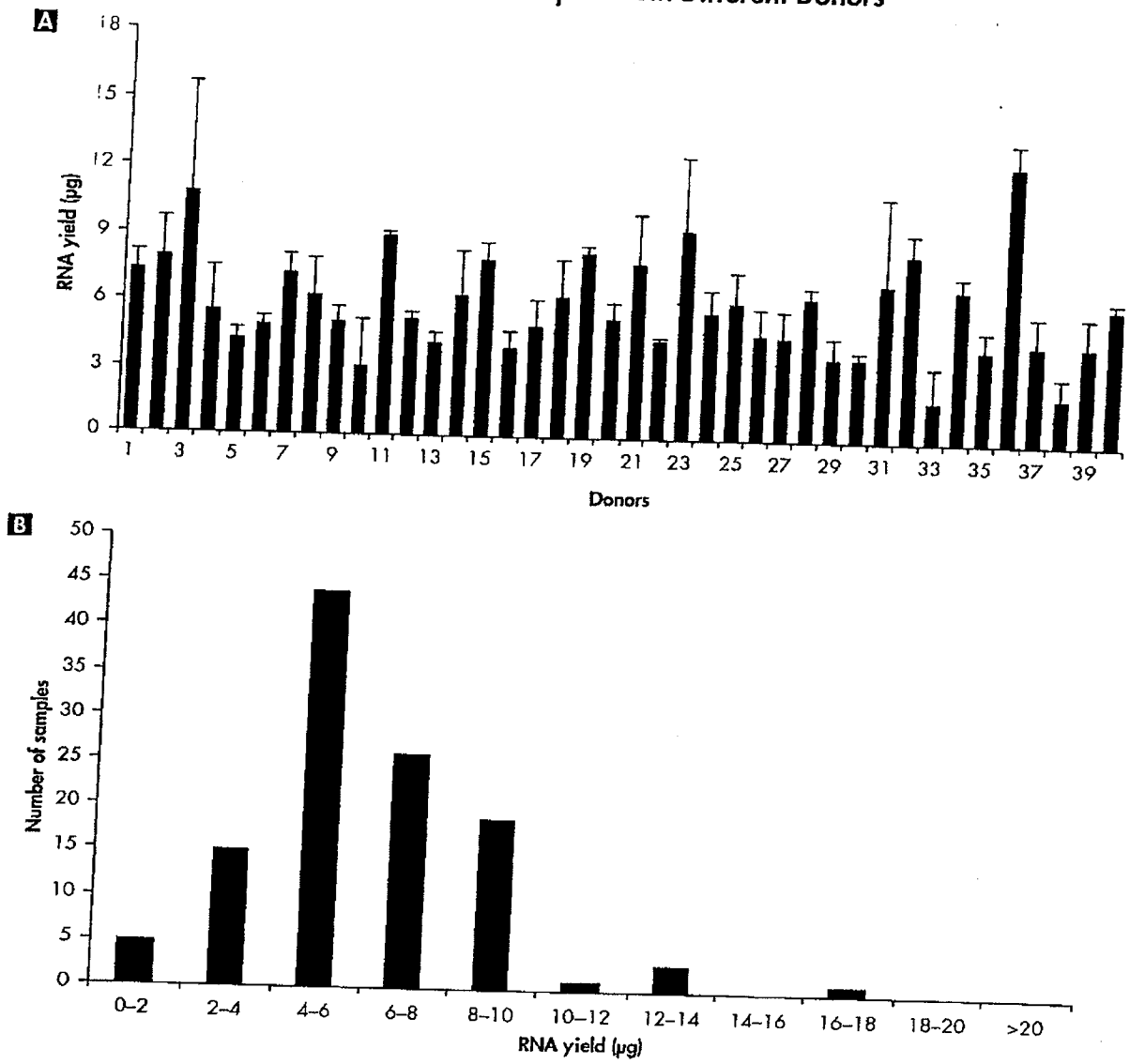


Figure 2. *Blood samples were collected in triplicate from 40 donors into PAXgene Blood RNA Tubes, and stored for <48 hours. RNA was purified using the PAXgene Blood RNA Kit with the DNase digestion step. Yields of purified RNA were determined by absorbance measurements at 260 nm in 10 mM Tris·HCl, pH 7.5.* **A** *Graph showing the average RNA yield obtained from each donor. Error bars indicate the standard deviation within triplicates.* **B** *Graph showing the distribution of RNA yields from the total of 120 blood samples.*

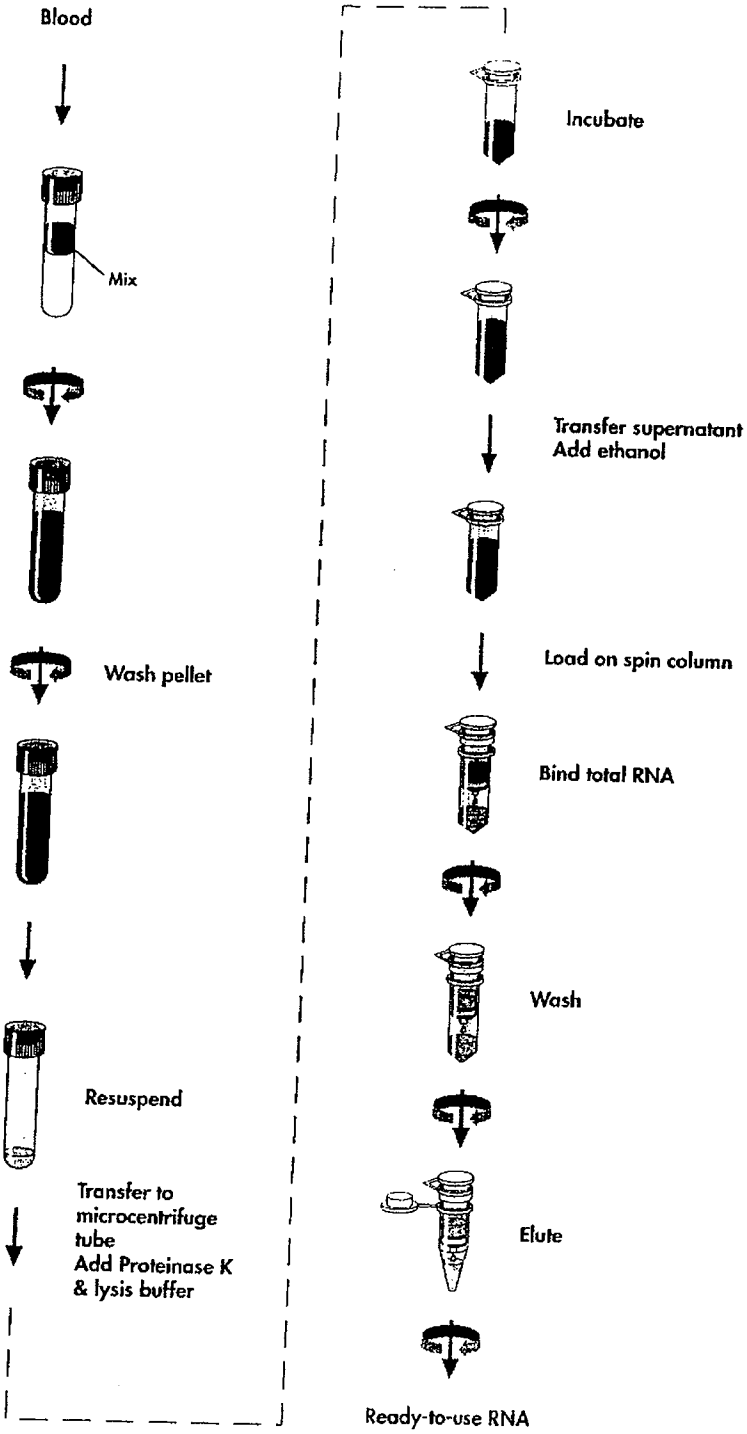
RNA purified using the PAXgene Blood RNA System is ready for use in a wide range of downstream applications, including:

- Nucleic acid amplification technologies, such as RT-PCR and NASBA®
- Quantitative RT-PCR, including TaqMan® technology
- Expression-array and expression-chip analysis
- Northern, dot, and slot blot analysis
- cDNA synthesis
- RNase and S1 nuclease protection
- Poly A+ RNA selection
- Primer extension

For further information about the PAXgene Blood RNA System, visit:

- **www.preanalytix.com/FAQ.asp** — provides frequently asked questions (FAQs) about the system
- **www.preanalytix.com/Tech_Notes.asp** — provides technical notes on the performance of the system

PAXgene Blood RNA Procedure



Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- PAXgene Blood RNA Tubes (QIAGEN, cat. no. 762115 [USA and Canada], 762105 [Japan], or 762125 [all other countries])
- Ethanol (96–100%)
- 1.5 ml or 2 ml microcentrifuge tubes
- Sterile, RNase-free pipet tips
- Variable-speed microcentrifuge with a rotor for 2 ml microcentrifuge tubes
- Centrifuge capable of attaining 3000–5000 x *g* and equipped with a swing-out rotor and buckets for holding PAXgene Blood RNA Tubes
- Shaker–incubator (e.g., Eppendorf® Thermomixer Compact), heating block, or water bath
- Vortex mixer
- Disposable gloves
- Optional: RNase-Free DNase Set (QIAGEN, cat. no. 79254)
- Optional: QIAshredder homogenizer (QIAGEN, cat. no. 79654 or 79656)
- Optional: RNeasy MinElute™ Cleanup Kit (QIAGEN, cat. no. 74204)
- Optional: Extra processing tubes (QIAGEN, Collection Tubes (2 ml), cat. no. 19201)

Protocol: Isolation of RNA from Whole Blood Collected into PAXgene Blood RNA Tubes

Important points before starting

- This protocol is for isolating cellular RNA from 2.5 ml human whole blood samples collected into PAXgene Blood RNA Tubes. The product circular supplied with the tubes provides instructions on drawing blood. PAXgene Blood RNA Tubes must be handled as follows:
  - After blood collection, tubes can be stored at room temperature (18–22°C) for up to 5 days (however, the exact storage time depends on the RNA species being examined; users must evaluate the number of days for which the tubes can be stored). For longer term storage, tubes can be stored at –20°C or –70°C for up to one year.
  - **If storing tubes at –20°C or below, do not place them in a tight Styrofoam rack, since they may crack.** Either place the tubes vertically in a loose wire rack or place the tubes horizontally in a plastic bag. If storing tubes at below –20°C, we recommend freezing at –20°C for 24 hours, and then transferring to colder storage.
  - Before starting the RNA purification procedure, ensure that the tubes have been incubated at room temperature for at least 2 hours in order to ensure complete cell lysis (overnight incubation may increase yields slightly in some cases). This incubation can be carried out either before or after storage at –20°C or below.
  - If tubes were immediately frozen after blood collection, then after removal from storage, first equilibrate to room temperature in a loose wire rack on the bench top (not in a water bath) for at least 2 hours (or overnight if desired), and then incubate at room temperature for an additional 2 hours. **Do not thaw the tubes at temperatures above 22°C.** After thawing, invert the tubes 10 times.
  - Frozen tubes are subject to breakage upon impact. To reduce the risk of breakage during shipment, frozen tubes should be treated in the same manner as glass tubes. Users must validate their own freezing and shipping protocol for PAXgene Blood RNA Tubes.
- Due to the sensitivity of nucleic acid amplification technologies, follow these precautions when handling PAXgene Blood RNA spin columns in order to avoid cross-contamination:
  - Carefully pipet the sample onto the spin column without moistening the rim.
  - Change pipet tips after each liquid transfer. We recommend using aerosol-barrier pipet tips.
  - Avoid touching the spin column membrane with the pipet tip.
  - To avoid cross-contamination after each vortexing step, we recommend briefly centrifuging the microcentrifuge tubes to remove drops from the inside of the lids.

- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
- Close the lid of the spin column before placing it in the microcentrifuge. Centrifuge as described in the procedure.
- Flow-through generated after centrifugation steps may contain hazardous materials and should be disposed of appropriately.
- Open only one spin column at a time, and take care to avoid generating aerosols.
- For efficient parallel processing of multiple samples, fill a rack with processing tubes to which spin columns can be transferred after centrifugation. Discard the used processing tubes containing flow-through, and place the new processing tubes containing spin columns directly in the microcentrifuge.
- PAXgene Blood RNA spin columns fit into most standard 1.5 and 2 ml microcentrifuge tubes. Additional processing tubes can be purchased separately from QIAGEN (Collection Tubes, cat. no. 19201).

- Perform all centrifugation steps of the procedure at room temperature (15–25°C).
- Sample-preparation waste, such as the supernatants from the first two centrifugation steps of the procedure (steps 2 and 3), may contain infectious material. We recommend classifying these supernatants as biological and chemical waste. These supernatants can be decontaminated by autoclaving and disposed of as chemical waste. Dispose of the waste in accordance with local, state, and federal regulations. Refer to safety information, page 6.

Things to do before starting

- If working with RNA for the first time, read Appendix B (page 22).
- Buffer BR4 is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- We strongly recommend using a shaker–incubator in step 5 of the procedure. Before starting the procedure, set the temperature of the shaker–incubator to 55°C. If a shaker–incubator is not available, use a heating block or water bath set to 55°C.
- Set a heating block or water bath to 65°C for use in step 15 of the procedure.

Procedure

1. **Centrifuge the PAXgene Blood RNA Tube for 10 min at 3000–5000 x *g* using a swing-out rotor with adapters for round-bottom tubes.**

   Note: To achieve complete cell lysis, the PAXgene Blood RNA Tube must be incubated for at least 2 h at room temperature prior to centrifugation.

2. **Remove the supernatant by decanting or pipetting, and discard. Add 5 ml RNase-free water to the pellet, and close the tube using a fresh secondary Hemogard closure.**

   If the supernatant is decanted, dry the rim of the tube with a clean paper towel.

3. **Thoroughly resuspend the pellet by vortexing, and centrifuge for 10 min at 3000–5000 x *g*. Remove the entire supernatant by decanting or pipetting, and discard.**

   Small debris remaining in the supernatant after vortexing but before centrifugation will not affect the procedure.

4. **Add 360 µl Buffer BR1, and vortex until the pellet is visibly dissolved.**

5. **Pipet the sample into a 1.5 ml or 2 ml microcentrifuge tube (not supplied). Add 300 µl Buffer BR2 and 40 µl Proteinase K. Mix by vortexing, and incubate for 10 min at 55°C using a shaker–incubator at 1400 rpm, a heating block, or a water bath.**

   To ensure complete mechanical disruption of genomic DNA, we strongly recommend using a shaker–incubator with the speed set to 1400 rpm. If using a heating block or water bath, vortex each sample once during the incubation. Vortex each sample for 10–20 s at maximum speed. Do not allow the temperature of the sample to decrease during vortexing.

   Note: Do not mix Buffer BR2 and Proteinase K together before adding them to the sample.

6. **If a shaker–incubator was used in step 5, centrifuge the sample for 3 min at maximum speed in a microcentrifuge, and transfer the supernatant to a fresh 1.5 ml or 2 ml microcentrifuge tube (not supplied). Proceed to step 7.**

   A minimum *g*-force of 10,000 x *g* is required.

   Note: The transfer of a small amount of debris remaining in the supernatant after centrifugation will not affect the procedure.

   If a heating block or water bath was used in step 5, vortex the sample for 30 s, centrifuge for 10 min at maximum speed in a microcentrifuge, transfer the supernatant to a fresh 1.5 ml or 2 ml microcentrifuge tube (not supplied), and proceed to step 7. Alternatively, pipet the lysate directly into a QIAshredder spin column (not supplied) placed in a 2 ml processing tube (supplied with the QIAshredder spin column), centrifuge for 3 min at maximum speed, carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 ml or 2 ml microcentrifuge tube (not supplied), and proceed to step 7.

   A minimum *g*-force of 10,000 x *g* is required.

   Note: The transfer of a small amount of debris remaining in the supernatant after centrifugation will not affect the procedure.

7. **Add 350 µl 96–100% ethanol. Mix by vortexing, and centrifuge briefly (1–2 s at ≤1000 x *g*) to remove drops from the inside of the tube lid.**

   Note: The length of this centrifugation step must not exceed 1–2 s, as this may result in pelleting of nucleic acids and reduced yields of total RNA.

8. **Add 700 µl sample to the PAXgene spin column placed in a 2 ml processing tube, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm). Place the PAXgene spin column in a new 2 ml processing tube, and discard the old processing tube containing the flow-through.**

9. **Add the remaining sample to the PAXgene spin column, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm). Place the PAXgene spin column in a new 2 ml processing tube, and discard the old processing tube containing the flow-through.**

   Optional: The RNase-Free DNase Set from QIAGEN provides convenient on-column DNase digestion during the RNA purification procedure. Further DNA removal is recommended for RNA applications that are sensitive to DNA contamination. If using the RNase-Free DNase Set, follow the procedure in Appendix A (page 20) after performing step 9 of this procedure.

10. **Add 700 µl Buffer BR3 to the PAXgene spin column, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm). Place the PAXgene spin column in a new 2 ml processing tube, and discard the old processing tube containing the flow-through.**

11. **Add 500 µl Buffer BR4 to the PAXgene spin column, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm). Place the PAXgene spin column in a new 2 ml processing tube, and discard the old processing tube containing the flow-through.**

    Note: Buffer BR4 is supplied as a concentrate. Ensure that ethanol is added to Buffer BR4 before use (see "Things to do before starting", page 14).

12. **Add another 500 µl Buffer BR4 to the PAXgene spin column. Centrifuge for 3 min at maximum speed to dry the PAXgene spin column membrane. Proceed to step 13, or to eliminate any chance of possible Buffer BR4 carryover, carry out the optional step described below, then proceed to step 13.**

    Note: After this washing step, the silica-gel membrane may be light or dark brown in color. This does not influence the quality of the RNA isolated, and has no effect on any downstream applications.

    Note: Residual Buffer BR4 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, and cause the flow-through containing Buffer BR4 to come into contact with the PAXgene spin column. Flow-through may also come into contact with the PAXgene spin column if the spin column and processing tube are not carefully removed from the rotor. In these cases, the optional step described below should be performed.

    Optional: Discard the old processing tube containing the flow-through, and place the PAXgene spin column in a new 2 ml processing tube (not supplied). Centrifuge for 1 min at full speed.

13. **Discard the old processing tube containing the flow-through from step 12. Place the PAXgene spin column in a 1.5 ml elution tube, and add 40 µl Buffer BR5 directly onto the PAXgene spin column membrane. Centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA. Do not discard the eluate.**

    To achieve maximum elution efficiency, evenly distribute Buffer BR5 over the entire surface of the membrane.

    Buffer BR5 will not interfere with downstream applications.

14. **Add another 40 µl Buffer BR5 directly onto the PAXgene spin column membrane. Centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute further RNA. Do not discard the eluate.**

    Optional: If eluted RNA is required in a volume of 40 µl, re-apply the eluate from step 13 to the PAXgene spin column again instead of using fresh 40 µl Buffer BR5. If even smaller volumes of eluted RNA are required, elute with 2 x 40 µl BR5, perform step 15, and use the RNeasy MinElute Cleanup Kit.

15. **Incubate the elutate for 5 min at 65°C in a heating block or water bath. After incubation, chill immediately on ice.**

    This incubation step denatures the RNA. Denatured RNA is essential for maximum efficiency in some downstream applications, such as RT-PCR, other amplification reactions, or cDNA synthesis. It is not necessary to denature the RNA again. The RNA remains denatured after freezing and thawing.

    To accurately quantify the RNA by absorbance measurement at 260 nm, we recommend diluting an aliquot of the eluate in 10 mM Tris·Cl, pH 7.5. Dilution in RNase-free water may lead to inaccurate quantification (Appendix D, page 25).

    Note: When quantifying RNA in Tris buffer, an absorbance of 1 at 260 nm (A_{260} = 1) is equivalent to an RNA concentration of 44 µg/ml.

    If analyzing RNA by gel electrophoresis, adding ethidium bromide to the gel only will lead to poor visualization of the RNA (Appendix E, page 27). For optimal visualization, add ethidium bromide to the loading buffer and mix with the RNA sample, incubate for 3–5 min at 65°C, chill on ice, and load onto a formaldehyde agarose gel.

Troubleshooting Guide

	Comments and suggestions
RNA degraded	
RNase contamination	Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be careful not to introduce any RNases during the procedure or later handling (see Appendix B, page 22).
RNA does not perform well in downstream experiments	
a) Salt carryover during elution	Ensure that Buffer BR4 is at room temperature.
b) Ethanol carryover during wash with Buffer BR4	Ensure that during the second wash with Buffer BR4 (step 12 of the procedure), the PAXgene spin column is spun at maximum speed for 3 min to dry the spin column membrane. After centrifugation, remove the PAXgene spin column from the processing tube carefully so that the flow-through does not come into contact with the spin column, causing carryover of ethanol. Continue with the optional centrifugation in step 12 of the procedure.
c) RNA eluate was not incubated at 65°C	Ensure that the RNA eluate is incubated at 65°C (step 15 of the procedure).
d) Buffer BR5 not used for elution	Be sure to use Buffer BR5 for eluting RNA.
Low RNA yield	
a) Less than 2.5 ml blood collected in the PAXgene Blood RNA Tube	Ensure that 2.5 ml blood is collected in the PAXgene Blood RNA Tube (see the product circular supplied with the PAXgene Blood RNA Tubes).
b) RNA concentration measured in water	RNA concentration must be measured in 10 mM Tris-Cl, pH 7.5 for accurate quantification (Appendix D, page 25).
c) Incubation with Proteinase K not carried out at the correct temperature	Ensure that the shaker–incubator used in step 5 is set to 55°C. If using a heating block or water bath, vortex the sample quickly during the incubation to minimize any cooling.
d) Cell debris transferred to the PAXgene spin column in steps 8 and 9 of the procedure	Avoid transferring large particles when transferring the supernatant in step 6 of the procedure (however, transfer of small debris will not affect the procedure).

	Comments and suggestions
e) Reduced or no supernatant after centrifugation in step 6 of the procedure (looks like a viscous pellet)	Genomic DNA was not completely disrupted. Vortex the sample and use the QIAshredder spin column as described in step 6 of the procedure.
f) Supernatant not completely removed in step 3 of the procedure	Ensure that the entire supernatant is removed. If the supernatant is decanted, remove drops from the rim of the tube by dabbing onto a paper towel.
g) After collection in the PAXgene Blood RNA Tube, blood is incubated for less than 2 hours	Incubate blood in the PAXgene Blood RNA Tube for at least 2 hours after collection. Incubation of the PAXgene Blood RNA Tube overnight may increase RNA yields slightly in some cases.
Low A_{260}/A_{280} ratio	
RNA diluted in water before purity is measured	Use 10 mM Tris·Cl, pH 7.5 to dilute RNA before measuring purity (Appendix D, page 25).

Appendix A: Optional On-Column DNase Digestion with the RNase-Free DNase Set

The RNase-Free DNase Set (QIAGEN, cat. no. 79254) provides efficient on-column digestion of DNA during the PAXgene RNA purification procedure. DNase is efficiently removed in subsequent wash steps.

Note: Standard DNase buffers are not compatible with on-column DNase digestion. Use of other buffers may affect binding of RNA to the PAXgene spin column membrane, reducing the yield and integrity of RNA in the eluate.

Lysis and homogenization of blood cells and binding of RNA to the PAXgene spin column membrane are performed according to the standard procedure. After washing with a reduced volume of Buffer BR3, RNA is treated with DNase I while bound to the spin column membrane. DNase I is removed by a second wash with Buffer BR3. Washing with Buffer BR4 and elution of RNA are then performed according to the standard procedure.

- **Prepare DNase I stock solution when using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. Do not vortex.**
- **Do not vortex the reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- The RNase-Free DNase Set contains RNase-free reagents and buffers for 50 RNA minipreps.

Procedure

Carry out steps 1–9 of the standard PAXgene RNA purification procedure (page 13). Instead of performing the wash with Buffer BR3 at step 10, follow steps 1–5 below.

1. **Add 350 µl Buffer BR3 to the PAXgene spin column. Centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm). Either discard the flow-through, or transfer the PAXgene spin column to a new processing tube (not supplied) and discard the old processing tube containing the flow-through.**
2. **Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.**

   Buffer RDD is supplied with the RNase-Free DNase Set.

   Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube. Do not vortex.

3. **Add the DNase I incubation mix (80 µl) directly onto the PAXgene spin column membrane, and incubate on the benchtop (20–30°C) for 15 min.**

   Note: Ensure that the DNase I incubation mix is added directly to the PAXgene spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

4. **Add 350 µl Buffer BR3 to the PAXgene spin column. Centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm). Place the PAXgene spin column in a new 2 ml processing tube, and discard the old processing tube containing the flow-through.**

5. **Continue with step 11 (the first wash with Buffer BR4) of the standard procedure.**

Appendix B: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Non-disposable plasticware

Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH,* 1 mM EDTA* followed by RNase-free water (see "Solutions", page 23). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

* *When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.*

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for four or more hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS),* thoroughly rinsed with RNase-free water, and then rinsed with ethanol*† and allowed to dry.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Carboxymethylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: PAXgene buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

* *When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.*

† *Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.*

Appendix C: Centrifuge Optimization

Performing the centrifugation steps of the PAXgene RNA purification procedure properly ensure optimal nucleic acid recovery. We highly recommend using a centrifuge with adjustable *g*-force. If such a centrifuge is not available, calculate *g*-force as follows:

$$rcf = 1.12 \times r \times (rpm/1000)^2$$

where rcf is the relative centrifugal force (in *g*), *r* is the radius of the rotor in centimeters, and rpm is the speed of the centrifuge in revolutions per minute.

Appendix D: Storage, Quantification, and Determination of Quality of Total RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in Buffer BR5.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be in the linear range of the spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260} = 1 ⇒ 44 µg/ml). This relation is valid only for measurements in 10 mM Tris·Cl, pH 7.5. Therefore, if it is necessary to dilute the RNA sample, this should be done in 10 mM Tris·Cl. As discussed below (see "Purity of RNA"), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be achieved by washing cuvettes with 0.1 M NaOH,* 1 mM EDTA* followed by washing with RNase-free water (see "Solutions", page 23). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 80 µl

Dilution = 10 µl of RNA sample + 140 µl 10 mM Tris·Cl, pH 7.5 (1/15 dilution).

Measure absorbance of diluted sample in a cuvette (RNase-free).

A_{260} = 0.3

Concentration of RNA sample = 44 x A_{260} x dilution factor
= 44 x 0.3 x 15
= 198 µg/ml

Total yield = concentration x volume of sample in milliliters
= 198 µg/ml x 0.08 ml
= 15.8 µg RNA

* *When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.*

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always calibrate the spectrophotometer with the same solution.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. To prevent any interference by DNA in RT-PCR applications, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. Alternatively, DNA contamination can be detected on agarose gels following RT-PCR by performing control experiments in which no reverse transcriptase is added prior to the PCR step or by using intron-spanning primers. For sensitive applications, such as differential display, or if it is not practical to use splice-junction primers, DNase digestion of the purified RNA with RNase-free DNase is recommended.

A protocol for optional on-column DNase digestion using the RNase-Free DNase Set is provided in Appendix A (page 20). The DNase is efficiently washed away in the subsequent wash steps. Alternatively, after the PAXgene procedure, the eluate containing the RNA can be treated with DNase. Following heat inactivation of the DNase, the RNA can be used directly in downstream applications.

Integrity of RNA

The integrity and size distribution of total RNA purified using the PAXgene Blood RNA System can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining (see Appendix E, page 27). The respective ribosomal bands (see Table 1) should appear sharp on the stained gel. 28S ribosomal RNA bands should be present with an intensity approximately twice that of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear of smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

Table 1. Size of Human Ribosomal RNAs

Human rRNA	Size (kb)
18S	1.9
28S	5.0

* *Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques* **22**, *474.*

† *Values up to 2.3 are routinely obtained for pure RNA (in 10mM Tris·Cl, pH 7.5) with some spectrophotometers.*

Appendix E: Protocol for Formaldehyde Agarose Gel Electrophoresis

The following protocol for formaldehyde-agarose (FA) gel electrophoresis is routinely used at PreAnalytiX and gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting). A key feature is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols (e.g., Sambrook, J. et al., eds. (1989) *Molecular cloning — a laboratory manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

1.2% FA gel preparation

To prepare FA gel (1.2% agarose) of size 10 x 14 x 0.7 cm, mix:

1.2 g	agarose*
10 ml	10x FA gel buffer (see composition below)
RNase-free water to 100 ml	

If smaller or larger gels are needed, adjust the quantities of components proportionately.

Heat the mixture to melt agarose. Cool to 65°C in a water bath. Add 1.8 ml of 37% (12.3 M) formaldehyde* and 1 µl of a 10 mg/ml ethidium bromide* stock solution. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x FA gel running buffer (see composition below) for at least 30 min.

RNA sample preparation for FA gel electrophoresis

Add 1 volume of 5x loading buffer (see composition below) per 4 volumes of RNA sample (for example 10 µl of loading buffer and 40 µl of RNA) and mix. Note that the loading buffer includes ethidium bromide. Adding ethidium bromide to the gel alone leads to poor visualization of the RNA when the RNA is prepared in Buffer BR5.

Incubate for 3–5 min at 65°C, chill on ice, and load onto the equilibrated FA gel.

Gel running conditions

Run gel at 5–7 V/cm in 1x FA gel running buffer.

* *When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.*

Composition of FA gel buffers

10x FA gel buffer

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)*
50 mM sodium acetate*
10 mM EDTA*
pH to 7.0 with NaOH*

1x FA gel running buffer

100 ml 10x FA gel buffer
20 ml 37% (12.3 M) formaldehyde
880 ml RNase-free water

5x RNA loading buffer

16 µl saturated aqueous bromophenol blue solution*†
80 µl 500 mM EDTA, pH 8.0
720 µl 37% (12.3 M) formaldehyde
2 ml 100% glycerol*
3084 µl formamide*
4 ml 10 x FA gel buffer
100 µl ethidium bromide (10 mg/ml)
RNase-free water to 10 ml

Stability: Approximately 3 months at 4°C

* *When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.*

† *To make a saturated solution, add solid bromophenol blue to distilled water. Mix and continue to add more bromophenol blue until no more will dissolve. Centrifuge briefly to pellet the undissolved powder, and carefully pipet the saturated supernatant.*

Ordering Information

Product	Contents	Cat. No.
PAXgene Blood RNA System — for blood collection, and RNA stabilization and isolation		
PAXgene Blood RNA Tubes (100)	100 PAXgene Blood RNA Tubes; to be used with the PAXgene Blood RNA Kit or PAXgene 96 Blood RNA Kit	762115* 762105† 762125‡
PAXgene Blood RNA Kit (50)	For 50 RNA preps: 50 PAXgene RNA Spin Columns, buffers, proteinase K, and processing tubes; to be used with PAXgene Blood RNA Tubes	762134
PAXgene Blood RNA Validation Kit (10)	For 10 RNA preps: 10 PAXgene Blood RNA Tubes, 10 PAXgene RNA Spin Columns, buffers, proteinase K, and processing tubes	762132
PAXgene 96 Blood RNA Kit (4)	For 4 x 96 RNA preps: 4 PAXgene 96 RNA Plates, 4 PAXgene 96 Filter Plates, buffers, proteinase K, RNase-Free DNase Sets, AirPore™ Tape Sheets, collection vessels; to be used with PAXgene Blood RNA Tubes	762331
Accessories		
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-free Buffer RDD, and RNase-free water for 50 RNA minipreps	79254
QIAGEN Proteinase K (10 ml)	10 ml (>600 mAU/ml, solution)	19133
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
RNeasy MinElute Cleanup Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	74204
QIAshredder (50)§	50 disposable cell-lysate homogenizers for use in nucleic acid minipreps, caps	79654

* *Canada and USA*
† *Japan*
‡ *All other countries*
§ *Other kit sizes available; please inquire*

Ordering Information

Product	Contents	Cat. No.
Related products		
PAXgene Blood DNA System — for blood collection and stabilization, followed by genomic DNA isolation		
PAXgene Blood DNA Tubes (100)	100 PAXgene Blood DNA Tubes; to be used with the PAXgene Blood DNA Kit	761115* 761105† 761125‡
PAXgene Blood DNA Kit (25)	For 25 DNA preps: buffers, protease, and processing tubes filled with lysis buffer; to be used with PAXgene Blood DNA Tubes	761133
PAXgene Blood DNA Validation Kit (10)	For 10 DNA preps: 10 PAXgene Blood DNA Tubes, buffers, protease, and processing tubes filled with lysis buffer	761132
Omniscript® RT Kit — for standard reverse transcription with any amount of RNA from 50 ng to 2 µg per reaction		
Omniscript RT Kit (50)§	For 50 reverse-transcription reactions: 200 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (contains 5 mM each dNTP), RNase-free water	205111
QIAGEN® OneStep RT-PCR Kit — for easy and sensitive one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)§	For 25 reactions: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep RT-PCR Buffer (containing 12.5 mM $MgCl_2$), dNTP Mix (containing 10 mM each dNTP), 5x Q-Solution, RNase-free water	210210

* *Canada and USA*
† *Japan*
‡ *All other countries*
§ *Other kit sizes available; please inquire*

Ordering Information

Product	Contents	Cat. No.
Products ordered from BD*		
Blood Collection Set	BD Vacutainer™ Safety-Lok™ Blood Collection Set: 21G, 0.75 inch needle, 12 inch tubing with luer adapter; 50 per box, 200 per case	367281† 367286‡
Needle Holder	Standard needle holder for 13 mm and 16 mm diameter; 1000 per case	364815† 364888‡
Serum Blood Collection Tube (discard tube)	BD Vacutainer Plus Plastic Blood Collection Tubes: serum tube, increased silica act clot activator, silicone-coated interior, 13 x 75 mm tube size, 4 ml draw volume, red BD Hemogard closure, paper label; 100 per pack, 1000 per case	367812

* *These blood collection accessories represent typical products that can be used with PAXgene Blood RNA Tubes. To find out more about these accessories, including how to order, visit* ***www.bd.com/vacutainer/products/venous.***

† *Canada and USA*

‡ *All other countries*

Notes

Notes

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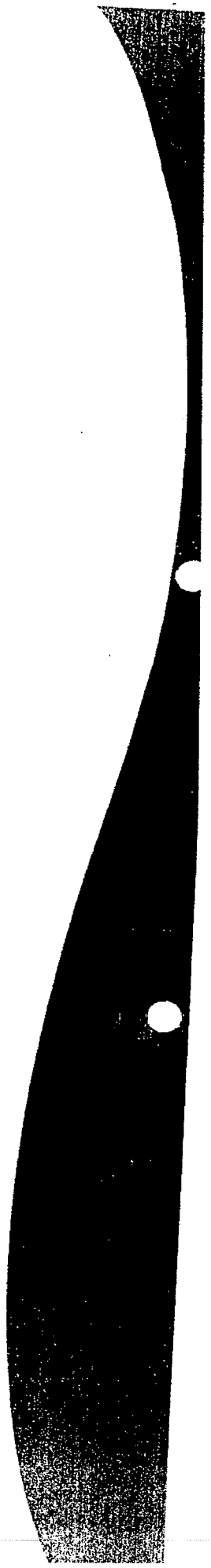

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